Hypertonic Resuscitation Modulates the Inflammatory Response in Patients With Traumatic Hemorrhagic Shock

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Objective: To determine the effect of resuscitation with hypertonic saline/dextran (HSD) on the innate immune response after injury.

Summary of Background Data: Hypovolemic shock causes a whole body ischemia/reperfusion injury, leading to dysregulation of the inflammatory response and multiple organ dysfunction syndrome. Hypertonicity has been shown to modulate the innate immune response in vitro and in animal models of hemorrhagic shock, but the effect on the inflammatory response in humans is largely unknown.

Methods: Serial blood samples were drawn (12, 24, 72 hours and 7 days after injury) from patients enrolled in a prospective, randomized, double-blind trial of traumatic hypovolemic shock, HSD (250 mL) versus lactated Ringer's solution (LR) as the initial resuscitation fluid. Neutrophil (PMN) CD11b/CD18 expression was assessed via whole blood FACS analysis with and without stimulation (fMLP 5 μmol/L or PMA 5 μmol/L). PMN respiratory burst was assessed using the nitro-blue tetrazolium assay. Monocytes stimulated with 100 ng LPS for 18 hours were assessed for cytokine production (TNF-α, IL-1B, IL-6, IL-10, IL-12).

Results: Sixty-two patients (36 HSD, 26 LR) and 20 healthy volunteers were enrolled. CD11b expression, 12 hours after injury, was increased 1.5-fold in patients resuscitated with LR compared with controls. Those resuscitated with HSD had a significant reduction in CD11b expression 12 hours after injury, compared with LR. There was no difference in respiratory burst early after injury. Monocytes from injured patients expressed lower levels of all cytokines in comparison to normal controls. Patients give HSD showed a trend toward higher levels of IL-1β and IL10 production in response to LPS, 12 hours after injury.

Conclusion: HSD resuscitation results in transient inhibition of PMN CD11b expression and partial restoration of the normal monocyte phenotype early after injury.

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Hypertonic solutions (7.5% saline ±6% dextran-70) have been investigated as alternative resuscitation strategies in critically injured patients.[1–6] Hypertonic resuscitation evokes an increase in serum osmolarity, which results in the redistribution of fluid from the interstitial and intracellular spaces to the intravascular space. This leads to rapid restoration of circulating intravascular volume, requiring a smaller volume of fluid compared with isotonic crystalloid solutions and leading to decreased accumulation of extravascular volume. Additionally, the osmotic effects of hypertonic fluids also reduce intracranial pressure in patients with head injury. Thus, this combination of increased systemic perfusion, which increases cerebral perfusion, along with a decrease in the intracranial pressure, which reduces brain swelling, minimizes the progression of secondary brain injury.

Despite evidence that has suggested benefit in early clinical trials and a series of subsequent meta-analyses that pointed toward improved outcome in patients treated with hypertonic resuscitation, hypertonic resuscitation strategies have not gained widespread acceptance in North America.[7–10] A renewed interest in this therapeutic approach has developed in response to in-depth basic laboratory investigations that have demonstrated that hypertonicity has significant effects on the innate and adaptive immune response that could mitigate the dysfunctional inflammatory response seen after traumatic injury.[11,12] These studies have suggested that the immunomodulatory effects of hypertonic fluids may lead to attenuation of organ injury and immune suppression seen after severe injury. The immunomodulatory effects of hypertonic fluids have been well defined in in vitro and in animal models, but only a small number of pilot studies have investigated these effects in humans.[13,14] We hypothesized that administration of hypertonic saline/dextran (HSD) would attenuate the initial neutrophil and monocyte proinflammatory response, which could then lead to reduced inflammatory organ injury. In addition, modulation of the early innate immune response could result in a less dramatic counter inflammatory response and thus a decrease in the risk of subsequent nosocomial infection and late organ failure. This study was designed to determine the effect of prehospital resuscitation with HSD on the activation of circulating neutrophils (PMN) and monocytes after severe injury.

METHODS

This study was conducted in conjunction with a blinded, randomized controlled trial of 7.5% saline/ 6% dextran 70 (HSD) versus lactated Ringer's solution (LR) as the initial resuscitation fluid administered in the prehospital setting. Eligible patients were those following blunt trauma, age ≥18 years, prehospital systolic blood pressure (SBP) <90 mm Hg, and altered mental status (GCS <15) in the prehospital setting. Exclusion criteria included: age <18 years, pregnancy, penetrating trauma, ongoing CPR, administration of >2000 mL of fluid prior to study drug availability, and transfer from a secondary hospital. Patients were randomized to receive 250 mL HSD or LR as the initial resuscitation fluid, followed by additional crystalloid as needed to support the blood pressure. HSD was used rather than 7.5% saline alone as preclinical studies have shown that the added dextran prolongs the duration of hypertonicity.[15,16] In addition, HSD is commercially available in Europe and thus the more clinically relevant fluid. The LR used contained L lactate alone rather than the racemic mixture of DL lactate. Patients were all transported to a single level 1 trauma center, and all care providers were blinded to the treatment assignment as the fluid was administered in identical 250-mL bags. Care providers and investigators were also blinded to serum sodium and chloride levels obtained during the first 12 hours after injury. All investigators remained blinded during the conduct of the laboratory studies. The primary endpoint for the clinical trial was the development of the acute respiratory distress syndrome (ARDS) within 28 days after injury, and the multiple organ dysfunction score (MODS) was collected as a secondary endpoint along with data regarding the development of nosocomial infections using CDC definitions.[17]

The laboratory studies reported here were conducted on a subset of the population enrolled in the trial based on availability of laboratory personnel. Patients selected for laboratory study were expected to survive >48 hours based on clinician judgment. Serial blood samples were obtained for these patients. The first sample was drawn within 12 hours of arrival, and subsequent samples were drawn at 24 hours, 72 hours, and 7 days after injury. Patients were enrolled in the trial under the Emergency Medicine Waiver of Informed Consent regulations, and informed consent was obtained from the patient or the legally authorized representative to continue in the study and participate in the blood sampling studies. Blood samples were also obtained from 20 healthy volunteers for comparison of normal values. This study was approved by the University of Washington Institutional Review Board for research involving human subjects.

Thirty milliliters of blood was drawn into a syringe coated with sodium citrate. PMNs and peripheral blood mononuclear cells (PBMCs) were then isolated by using Ficoll Paque density centrifugation. PMNs were then assessed for respiratory burst activity, as described below, using the nitro-blue tetrazolium assay. PBMCs were plated at 1 million cells/mL and stimulated with lipopolysaccharide (LPS) (100 ng/mL). Supernatants were harvested after 18 hours and stored at −70°C for subsequent cytokine analysis. During the latter portion of the study, a sample of whole blood was set aside for analysis of PMN expression of CD11b/CD18 using flow cytometry.

Assays of PMN Activation

Respiratory Burst

PMNs were isolated by Ficoll-Paque (Amersham Pharmacia, Piscataway, NJ) density gradient centrifugation followed by a dextran separation. Cells were plated at 2×10^5 cells in a 96-well plate in HBSS and allowed to adhere for 30 minutes. Cells were then treated with 100 nmol/L of iodoacetamide (negative control) (Sigma, St. Louis, MO) or stimulants (25 nmol/L phorbol-myristate-acetate [PMA], 1 μmol/L formyl-methionyl-leucyl-phenylalanine [fMLP]) (Sigma) in a 1-mg/mL solution of nitro-blue tetrazolium (Sigma).[18,19] The plate was then placed in the SPECTRAmax Plus plate reader (Molecular Devices, Sunnyvale, CA) at 37°C and OD readings at 550 nm were taken every 15 minutes for 75 minutes for a kinetic read. V_{max} was calculated using the SOFTmax software (Molecular Devices).

CD11b Surface Expression

Whole blood samples were stimulated with PMA (5 μmol/L) or fMLP (5 μmol/L) with unstimulated controls for 30 minutes. Cells were then labeled with anti-CD11b-FITC monoclonal antibody or isotype control antibody (IgG_2) (BD Bioscience, San Jose, CA) for 30 minutes. After treatment with FACS lysing solution (BD Bioscience), cells were fixed in paraformaldehyde for subsequent FACS analysis. The PMN population was identified by forward and side scatter using a FACScan flow cytometer (BD Bioscience). The degree of CD11b expression was determined by mean fluorescence as analyzed by the CELL Quest software.

Assays of Monocyte Activation

PBMCs were isolated, plated, and stimulated with or without LPS as described above. After 18 hours of incubation, supernatants were harvested and stored for cytokine analysis (−70°C). The production of tumor necrosis factor (TNF-α), and interleukins 1β, 6, 10, and 12 were determined using the Beadlyte human multicytokine detection system kit (Upstate, Lake Placid, NY). Plates were read on a Luminex 100 multiple analyte profiling system (MiraiBio, Alameda, CA) and analyzed using the MasterPlex QT software. Results are expressed as pg/mL.

Statistical Analysis

Comparison of demographic, injury severity, and clinical outcome data between the patient groups was assessed using the χ^2 test for categorical variables and the Student *t* test for continuous variables,. Comparison of the laboratory data between the treatment groups was performed using ANOVA with Bonferroni correction using the statistical software, STATA (Stata Inc, College Station, TX). Significance was considered as $P < 0.05$.

RESULTS

Between October 2003 and August 2005, there were 209 patients enrolled in the randomized clinical trial of HSD resuscitation of whom 62 had serial blood samples drawn. Thirty-six had been randomized to HSD and 26 to LR as the initial resuscitation fluid administered in the prehospital setting. All samples had assessment of PMN respiratory burst

TABLE 1. Demographics, Injury Severity, and Clinical Outcome Data

	HSD (n = 36)	LR (n = 26)	*P*
Mean age (yr)	378 ± 15	36 ± 16	0.62
% male	69	85	0.17
Admission Na (mEq/dL)	146 ± 3	139 ± 3	<0.001
Mean ISS	25 ± 13	31 ± 10	0.05
ISS >25	20 (56%)	20 (77%)	0.08
Mean TRISS	69 ± 30	65 ± 37	0.64
Mean PRBC 1st 24 hr (units)	4.5 ± 6.1	6.7 ± 12	0.35
>10 units PRBC 1st 24 hr	5 (14%)	5 (19%)	0.57
Mean ICU days	10 ± 8	10 ± 8	1.0
Ventilator free days	18 ± 9	18 ± 9	1.0
Any nosocomial infection	9 (25%)	8 (31%)	0.61
Worst MODS	6.2 ± 4.1	7.6 ± 6.1	0.25
ARDS	13 (36%)	10 (38%)	0.85
Mortality	3 (8%)	3 (11.5%)	0.67

ISS indicates Injury Severity Score; PRBC, packed red blood cells; ICU, intensive care unit; MODS, multiple-organ dysfunction score; ARDS, acute respiratory distress syndrome.

TABLE 2. The Inflammatory Response of Cells From Normal Volunteers

Assay	Mean (SD)
NBT (n = 6, V_{max})	
Unstimulated	1.0 (0.4)
fMLP	1.7 (0.5)
PMA	2.0 (0.4)
CD11b (n = 6, mean fluorescence)	
Unstimulated	378 (158)
fMLP	2083 (510)
PMA	3314 (859)
PBMC cytokine response (n = 20, pg/mL)	
TNF-unstimulated	175 (177)
TNF-LPS	5542 (474)
IL-1-unstimulated	98 (66)
IL-1-LPS	11,619 (11015)
IL-6-unstimulated	648 (794)
IL-6-LPS	6234 (631)
IL-10-unstimulated	23 (6)
IL-10-LPS	1115 (867)
IL-12-unstimulated	24 (3)
IL-12-LPS	154 (157)

NBT indicates nitro blue tetrazolium; fMLP, formyl-methionyl-leucyl-phenylalanine; PMA, phorbol-myristate-acetate; PBMC, peripheral blood mononuclear cells; LPS, lipopolysaccharide; IL, interleukin; TNF, tumor necrosis factor.

and monocyte cytokine production. Whole blood FACS analysis of CD11b expression was performed on samples from the last 15 patients enrolled. Blood samples were also obtained from 6 healthy volunteers for assessment of respiratory burst and CD11b expression and from 20 healthy volunteers for monocyte cytokine expression. The demographics, injury severity, and clinical outcome of the patients included are illustrated in Table 1. Of note, there was a higher mean injury severity score (ISS) in the patients in the LR group. As expected, there was a significant difference in the sodium level measured at hospital admission with a mean of 146 mEq/dL for the HSD group and 139 mEq/dL for the LR group ($P < 0.001$). It is not possible to estimate the true peak serum sodium for these patients as there is inherent variability in the timing of the initial serum sodium based on differences in prehospital transport times, amount of crystalloid received in the prehospital setting, and the rate of ongoing hemorrhage prior to arrival. There were no significant differences in the clinical outcome parameters between the treatment groups. For comparison to the patient data, the results of these studies in normal volunteers are illustrated in Table 2. Of note, there was significant individual variability in the monocyte cytokine response to endotoxin among the cells from healthy volunteers as demonstrated by the wide standard deviations.

PMN Activation

As illustrated in Figure 1, there were no significant differences in respiratory burst activity between the treatment groups with the exception of the PMA stimulated cells 7 days after injury ($P = 0.03$). The values observed in response to both fMLP and PMA stimulation were consistent with those seen in healthy volunteers. CD11b expression, on the other hand, was significantly higher in patients than in healthy volunteers. As shown in Figure 2A, CD11b expression 12 hours after injury was increased 1.5-fold in patients resuscitated with LR compared with normal controls. Those resuscitated with HSD had a significant reduction in CD11b expression 12 hours after injury that approached the response seen in healthy volunteers (Table 2). There were no significant differences between the groups beyond 12 hours after injury. As shown in Figure 2B and C, PMNs from both treatment groups responded to both PMA and fMLP in a similar fashion with a significantly greater response seen in the PMA-treated cells from the LR treatment group 12 hours after injury.

Monocyte Activation

As illustrated in Figure 3, circulating PBMCs from patients produced very low levels of cytokines at every time point when not stimulated ex vivo. The cytokine response to LPS of the normal control volunteers is shown in Table 2. Following LPS stimulation, production of all cytokines was lower in patients than the response observed in cells from healthy volunteers (Table 3). Patients treated with HSD tended to have cytokine responses to LPS that were closer to normal values.

As shown in Figure 3A and B, there were no differences between the treatment groups in the production of TNF-α or IL-6 at any time point. LPS-induced IL-1β production was higher in the HSD group at both the 12- and 24-hour time points, but this difference did not reach statistical significance ($P = 0.07$). LPS-induced IL-10 production 12 hours after injury was less than half the level see in cells from healthy volunteers and there was a trend toward higher IL-10 levels in the HSD-treated group ($P = 0.17$). LPS-induced IL-10 production increased over time in the LR

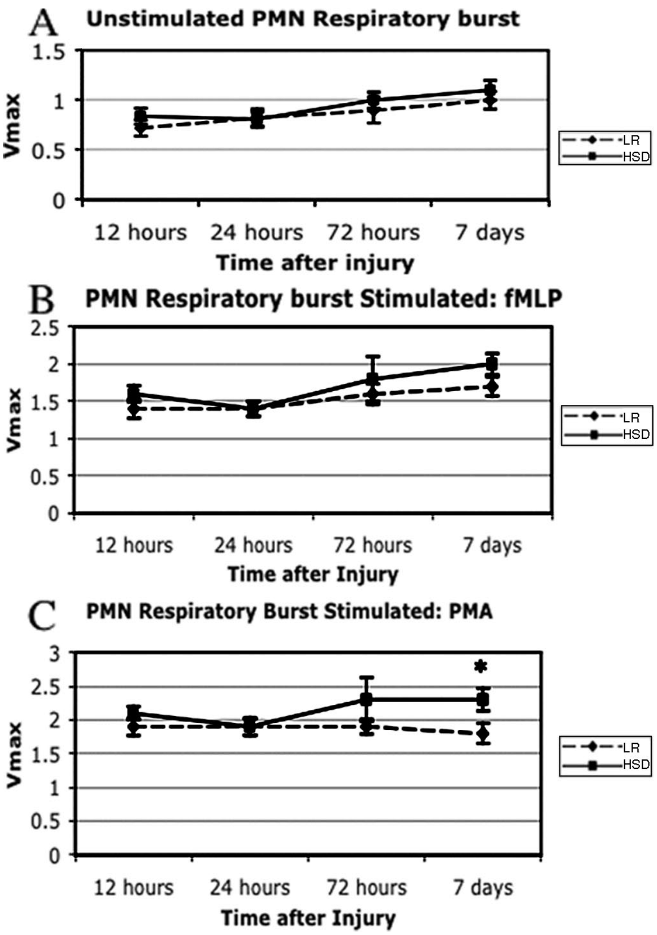

FIGURE 1. PMN respiratory burst. Serial blood samples obtained from injured patients with hypotension treated with HSD versus LR as the initial resuscitation fluid, administered in the prehospital setting. PMNs were isolated and assessed using a nitro-blue tetrazolium assay for respiratory burst activity. Cells were either unstimulated (A) or stimulated with fMLP (B) or PMA (C), in vitro. Data expressed as V_{max}. Error bars represent standard error of the mean. The V_{max} for cells from healthy volunteers was: unstimulated, 1.0 (SD 0.4); fMLP, 1.7 (SD 0.5); PMA, 2.0 (SD 0.4). $^*P < 0.05$.

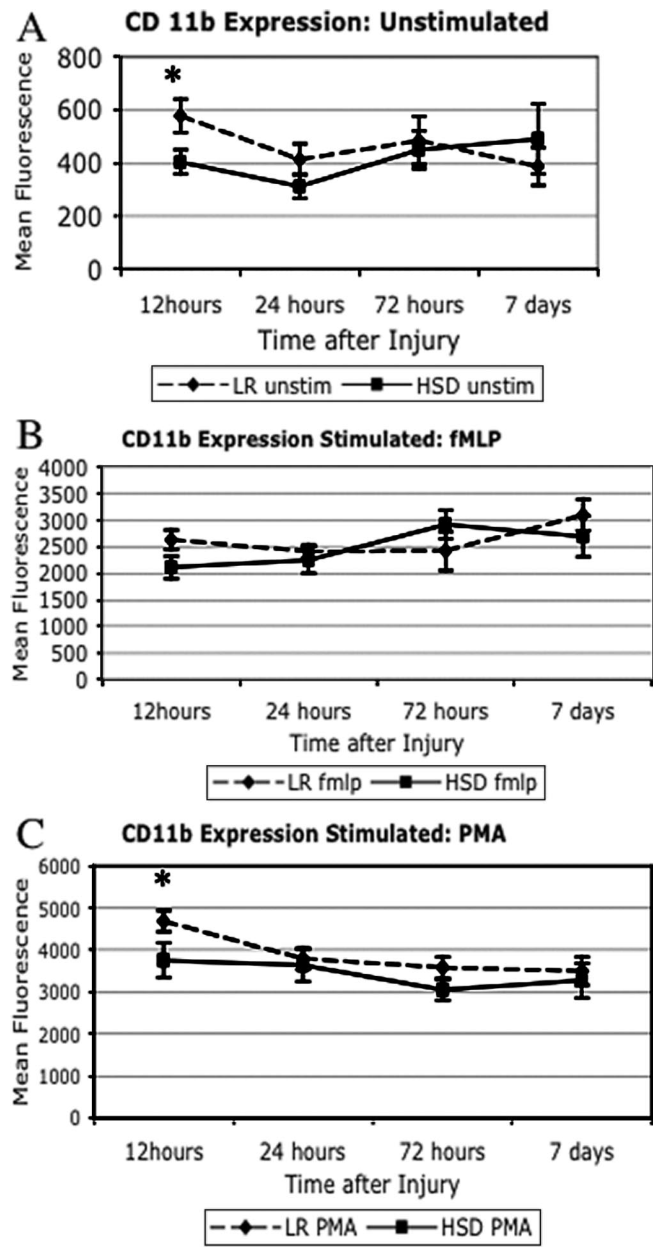

FIGURE 2. PMN CD11b expression. Serial blood samples obtained from injured patients with hypotension treated with HSD versus LR as the initial resuscitation fluid, administered in the prehospital setting. Whole blood FACS analysis was performed to evaluate PMN CD11b expression. A, The level of CD11b expression of PMNs after injury. B and C, CD11b expression in response to stimulation with fMLP and PMA, respectively. The mean fluorescence for cells obtained from healthy volunteers was: unstimulated, 378 (SD 158); fMLP, 2083 (SD 510); PMA, 3314 (SD 859). Error bars represent standard error of the mean. $^*P < 0.05$.

group but remained relatively constant in the HSD group. IL-12 levels were also measured but were very low under all conditions (data not shown).

DISCUSSION

Dysfunctional regulation of the innate immune response after injury contributes to the development of inflammatory organ injury including ARDS and MODS.[20] The effect of hypertonicity on the activation of inflammatory cells has been widely studied both in vitro and in animal models. In vitro studies of PMN function have demonstrated that hypertonicity reduces PMN-mediated bacterial killing, reactive oxygen species generation, and degranulation.[21–23] In addition, hypertonicity leads to an extensive shedding of CD62L from the PMN cell surface while preventing the up-regulation of CD11b/CD18,[24–26] thus rendering these cells unable to roll or adhere to the endothelium. These in vitro findings have been confirmed in animal models of lung injury. Hypertonic resuscitation has been shown to significantly attenuate inflammatory lung injury in a two-hit animal model consisting of hemorrhagic shock with reperfusion followed by and intra-

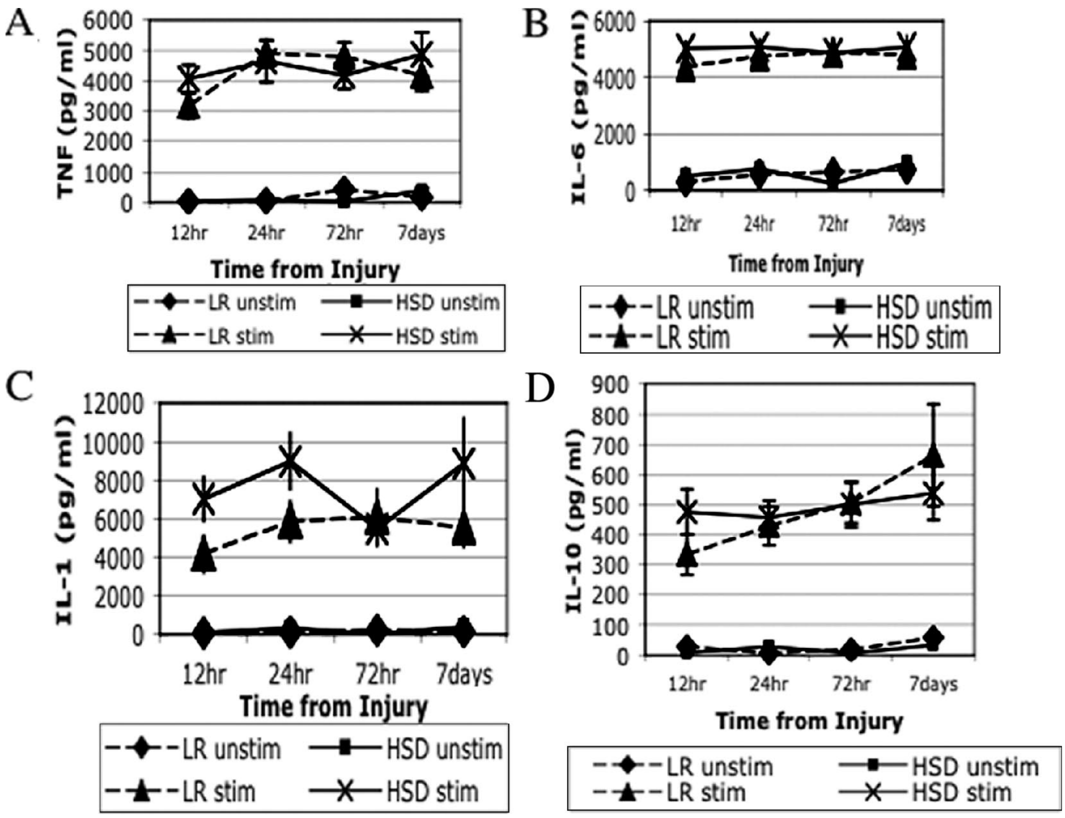

FIGURE 3. PBMC cytokine production. Serial blood samples obtained from injured patients with hypotension treated with HSD versus LR as the initial resuscitation fluid, administered in the prehospital setting. PBMCs were isolated, plated, and treated with or without LPS (100 ng/mL) for 18 hours. Cytokine levels from the supernatant were determined using a Luminex assay. A, TNF production. B, IL-6. C, IL-1. D, IL-10. Error bars represent standard error of the mean.

tracheal endotoxin challenge.[27] Lung injury was also attenuated by hypertonic resuscitation in a hemorrhagic shock model by reducing hemorrhage-induced activation of PMN oxidative burst.[28]

Although these observations have been very consistent in in vitro and in animal models, only one previous study has evaluated PMN function in injured patients receiving hypertonic saline. This study by Rizoli et al enrolled 24 patients with hypovolemic shock who were given HSD or LR in the emergency department with blood drawn prior to infusion and serially over the first 24 hours after infusion.[14] Similar to reports from experimental models, they found that hypertonic saline abolished shock-induced CD11b up-regulation while causing extensive CD62L shedding. Similar to our findings, the differential response in CD11b expression was most marked in PMN obtained from patients in hypovolemic shock, which were unstimulated ex vivo. In their study, inhibition of CD11b expression extended to 24 hours, whereas we observed a significant difference at only 12 hours after injury. As the HSD in our study was administered in the prehospital setting rather than the ED, the effects may have been diluted by additional fluid given after dosing. The Rizoli et al study[14] did not assess PMN respiratory burst, and we did not identify any difference between the groups with the exception of the PMA stimulated cells 7 days after injury. The clinical significance of this difference under only one stimulatory condition is unclear. The effects of hypertonicity on respiratory burst may be more immediate and thus may have been more evident at earlier time points.

TABLE 3. Comparison of PBMC Cytokine Response to LPS Between Patients (12 Hours After Injury) and Normal Controls

	Normal; LPS (n = 20)	12-Hour HSD; LPS (% of NL) (n = 36)	12-Hour LR; LPS (% of NL) (n = 26)
TNF-α (pg/mL)	5542	4063 (73%)*	3195 (58%)*
IL-1β (pg/mL)	11619	7026 (60%)*	4103 (35%)*
IL-6 (pg/mL)	6234	5034 (81%)*	4752 (76%)*
IL-10 (pg/mL)	1115	476 (43%)*	334 (30%)*
IL-12 (pg/mL)	154	53 (34%)*	33 (21%)*

*$P < 0.05$ versus normal control.
LPS indicates lipopolysaccharide; HSD, hypertonic saline/dextran; LR, lactated Ringer's solution; TNF, tumor necrosis factor; IL, interleukin.

The effect of hypertonic saline on monocyte/macrophage activation is less well defined. Hypertonic preconditioning has been shown to inhibit the macrophage responsiveness to inflammatory stimuli, such as endotoxin.[29] These studies demonstrated a significant reduction in TNF-α production and activity in response to endotoxin following hypertonic saline pretreatment. Recently, it has been demonstrated that circulating monocytes exist as 2 major subsets that have distinct phenotypical and functional properties.[30] The major subset is strongly CD14-positive, but negative for CD16 (CD14+CD16− monocyte). The other subset comprises approximately 10% of circulating monocytes in healthy individuals. It is weakly CD14-positive but also CD16-positive (CD14+CD16+ monocyte). This subset is considered to be pro-inflammatory since it readily expresses TNF-α and IL-1β but fails to produce significant amounts of IL-10.[31,32] This change in monocyte phenotypic differentiation toward a CD14+CD16+ subset has been demonstrated to occur following trauma, and to be prognostic for the development of sepsis and multiple organ dysfunction.[33,34] In the study of ED administration of HSD by Rizoli et al,[14] they observed that trauma was associated with a drop in the strongly CD14 positive cells (CD14++ monocytes) and a marked increase in the weakly CD14 positive CD14+CD16+ subset that has a pro-inflammatory role. Patients treated with HSD demonstrated a modest increase in the CD14++CD16− monocytes subset at early time points and a significant reduction in the pro-inflammatory CD14+CD16+ subset throughout the observation period. These changes in the monocyte subset distribution were paralleled by shifts in monocyte cytokine production profiles based on intracellular staining. Specifically, HSD markedly increased the production of IL-10 and IL-1ra, anti-inflammatory mediators produced by the CD14++CD16− subset, and at the same time dramatically reduced expression of TNF-α produced by the pro-inflammatory CD14+CD16+ subset. These changes were only observed within the individual cell subsets studied using whole blood FACS analysis. Our studies of monocyte activation were based on isolation of PBMC and subsequent cytokine production ex vivo. Although we observed a trend toward higher IL-10 and IL-1β levels 12 hours after injury in the HSD group, this did not reach statistical significance. As our population of monocytes represents a mixture of the subsets described above, it is likely that subtle changes in monocyte cytokine responsiveness can only be observed by direct examination of the individual subsets.

Interestingly, all the cytokine responses we observed from injured patients were lower than levels produced by cells from healthy volunteers, suggesting a general suppression of monocyte responsiveness following severe injury. Previous studies have also reported inhibition of PBMC cytokine production after injury.[35,36] These authors have reported decreased production of IL-1β for up to 10 days after severe trauma and increased production of the immunosuppressive prostaglandin PGE_2. Circulating levels of plasma cytokines, aside from IL-6, have also been consistently low after injury but may not be reflective of activation of cells at the tissue level.[37] In addition, monocyte cell-associated levels of TNF-α have been reported as elevated after injury, but secretion of TNF-α may be impaired by high levels of PGE_2.[36] Our data reveal consistent inhibition of cytokine production by the circulating monocyte after injury, which is partially restored by HSD treatment. Further study of the distribution of monocyte subsets and the relevance of cell associated versus secreted cytokines are needed. In addition, comparison of the response of the tissue-fixed macrophage versus the circulating monocyte would help determine the relevance of these changes to end organ damage.

A primary limitation to these studies is the fact that we could not include all patients enrolled in the clinical trial; thus, a selection bias is possible. Patients were included based on the availability of laboratory personnel and the expectation that they would survive >48 hours. Moribund patients were not enrolled, as this population is not likely to survive to suffer the late consequences of early changes in the innate immune response. An additional limitation was that the clinical trial was closed early for futility and thus the sample size for the CD11b studies was limited. Even with this small sample size, we were able to detect a significant difference at the 12-hour time point, but the addition of more patients to this analysis may have allowed us to detect a difference at later time points as well. The addition of samples from time points earlier than 12 hours might have allowed detection of greater differences between the HSD and LR groups as most patients had normalized their sodium levels by 12 hours. Differences in PMN respiratory burst activity may also have been more evident at earlier time points. Although the difference in admission serum sodium concentration was significant and similar to that seen in previous trials,[14] a more prolonged elevation in sodium levels may be required to result in long lasting immunologic effects. In a study by Murao et al, repeated dosing of 7.5% saline resulted in more effective reduction in lung injury in a mouse model of hemorrhagic shock.[38] Other studies, however, have demonstrated adverse metabolic consequences of repeated dosing in uncontrolled hemorrhage models.[39,40] Repeated dosing or a strategy to maintain hypertonicity over a longer period of time warrants further investigation.

CONCLUSION

These data confirm the early up-regulation of PMN CD11b expression after severe injury in comparison to healthy volunteers. We demonstrate inhibition in PMN CD11b expression following HSD treatment. We also demonstrate a significant suppression of the circulating monocyte response to LPS, which begins as early as 12 hours after injury and is partially restored by HSD treatment at the time of reperfusion. Further study of the immunologic effects of hypertonic resuscitation will help to further define the clinical utility of this resuscitation strategy.

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